



PATENT
UTXC:504

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**INHIBITION OF Bcl-2 PROTEIN EXPRESSION
BY LIPOSOMAL ANTISENSE OLIGODEOXYNUCLEOTIDES**

By:

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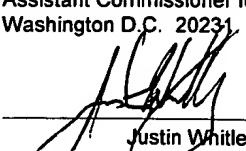
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BACKGROUND OF THE INVENTION

A. Field of the Invention

The present invention relates to the field of cancer therapy, specifically, the treatment of follicular lymphoma. More particularly, these treatments involve the use of antisense oligodeoxynucleotides and liposomal formulations thereof.

B. Related Art

Bcl-2 has been linked to a wide variety of diseases such as hematologic malignancies, both leukemias and lymphomas, including follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias (Campos *et al.*, 1994); solid tumors like those associated with breast, prostate and colon cancer; and immune disorders. One particular Bcl-2-related disease is Follicular non-Hodgkin Lymphoma (FL). FL is the most common lymphoid malignancy in Europe and the United States. Typically it is an indolent, low grade disease consisting of an accumulation of small, resting B cells. Although the response to chemotherapy is initially good, relapses are inevitable with the transformation to a more aggressive histological type and the development of drug resistance (Aisenberg, 1995; Johnson *et al.*, 1995). In over 90% of FL patients, a t(14;18) translocation is found, which results in the juxtaposition of the *bcl-2* gene from chromosome 18q21 with the immunoglobulin heavy chain gene locus on chromosome 14q323 (Tsujimoto *et al.*, 1985; Graninger *et al.*, 1987). As a consequence, the *bcl-2* gene is under the influence of immunoglobulin heavy chain enhancer, and the Bcl-2 protein is overexpressed (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1987). Bcl-2 tumorigenic potential is related to its capacity of interfering with physiological death responses, thereby enhancing the longevity of the cell (Nuñez *et al.*, 1990). The Bcl-2 protein blocks apoptotic stimuli such as growth factor deprivation, radiation, heat-shock, virus, and most of the chemotherapeutic agents (Reed, 1995; Hockenbery *et al.*, 1990). In *bcl-2*-Ig-transgenic mice, a polyclonal follicular lymphoproliferation consisting of an expansion of mature B lymphocytes is initially

observed (McDonnell *et. al*, 1989). Subsequently, a monoclonal high grade large immunoblastic type lymphomas develop with 50% of them presenting rearrangement of C-MYC. This suggests that a second genetic alteration is necessary for the development and progression of malignant lymphoma (McDonnell and Korsmeyer, 1991).

5 Recently, an expanding family of Bcl-2-related proteins has been identified. This includes Bax, Bcl-X_L, Bcl-X_S, Bad, Bak, Mcl-1, A-1, and several open reading frames in DNA viruses (Oltvai *et. al*, 1993; Boise *et. al*, 1993; Yang *et. al*, 1995; Chittenden *et. al*, 1995; Kiefer *et. al*, 95; Kozopas *et. al*, 1993; Lin *et. al*, 1993; Pearson *et. al*, 1987; Neilan *et. al*, 1993). Membership in the Bcl-2 family of proteins is principally defined by
10 homology within the BH1 and BH2 domains, which help regulate dimerization between the members (Sato *et. al*, 1994). Bax, which shares 21% amino-acid identity with Bcl-2, can bind to Bcl-2 protein and neutralize its ability to block cell death. Thus, the ratio of Bcl-2 to Bax is thought to determine the cell's susceptibility to death following an apoptotic stimulus (Oltvai *et. al*, 1993; Yin *et. al*, 1994).

15 Phosphodiester antisense oligodeoxynucleotides complementary to specific sequences of the translation-initiation site of Bcl-2 mRNA are able to inhibit the production of the Bcl-2 protein and the growth of t(14;18) translocation bearing cells (Kitada *et. al*, 1993). However, the therapeutic use of antisense oligonucleotides has been hampered by their low cellular uptake and their rapid degradation by nucleases and other
20 serum or cellular components. Phosphorothioate oligonucleotides, which are resistant to nuclease degradation, were found to inhibit FL cell growth at concentrations 10 times lower than phosphodiester oligonucleotides (Reed *et. al*, 1990a; Cotter *et. al*, 1994). However, this approach suffers from low cellular uptake of the oligonucleotides. For example, Reed *et. al* had to use concentrations of greater than 25µM of phosphorothioates
25 to achieve 50% growth inhibitions of cell lines derived from B-cell lymphomas, such as 697 and Su-Dhl-4 cells. Liposomal incorporation has led to enhanced uptake of oligonucleotides into leukemic cells (Akhtar *et. al*, 1991; Tari *et. al*, 1994). The use of cationic lipids by Reed *et. al* to deliver phosphorothioate antisense oligonucleotides

The term "lipids" as used in this specification and the claims denotes any form of both naturally occurring and synthetic lipids or liposomes. They are fatty substances and are well-known by those of skill in the art. The lipids of the present invention are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either size or shape. The lipid may advantageously be comprised of the lipid dioleoylphosphatidylcholine, however other lipids such as other phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines may also be employed.

In yet another embodiment, there is provided a composition comprising a polynucleotide that hybridizes to a Bcl-2-encoding polynucleotide.

In still yet another embodiment, there is provided a composition comprising an expression construct that encodes a polynucleotide that hybridizes to a Bcl-2-encoding polynucleotide, wherein said first polynucleotide is under the control of a promoter that is active in eukaryotic cells.

This invention also comprises a method for inhibiting proliferation of a cancer cell comprising contacting said cancer cell with a composition comprising at least a polynucleotide that hybridizes to a Bcl-2-encoding nucleic acid. This method may be applied advantageously to a cancer cell that is a lymphoma cell or, more specifically, a follicular lymphoma cell. The composition may comprise a lipid which is associated with the polynucleotide, such as a polynucleotide encapsulated in a liposome. In a specific embodiment, the contacting takes place in a patient. The patient may be a human. The composition may advantageously be delivered to a human patient in a volume of 0.50-10.0 ml per dose or in an amount of 5-30 mg polynucleotide per m². In a particular regimen, the composition is administered 3 times per week for 8 weeks.

This invention relates to antisense technology that may be employed to treat Bcl-2-associated disease. In one embodiment it encompasses a composition comprising a

polynucleotide that hybridizes to a Bcl-2-encoding polynucleotide and a lipid associated with the polynucleotide. The polynucleotide may be an oligonucleotide having a length of between about 8 and about 50 bases. However, oligonucleotides of other lengths may also be useful. The polynucleotide may also hybridize to the translation initiation site of Bcl-2 mRNA. An example of a useful polynucleotide is an oligonucleotide comprising the sequence CAGCGTGCGCCATCCTTC (SEQ ID NO:1).

Compositions of the present invention also include compositions wherein liposomes are formed from the lipid. In some cases, it may be useful to have a composition in which the polynucleotide is encapsulated in the liposome. Lipids that are useful in the present invention include phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines, one example being the lipid dioleoylphosphatidylcholine.

An embodiment of this invention is a composition comprising an expression construct that encodes a polynucleotide that hybridizes to a Bcl-2-encoding polynucleotide, wherein said polynucleotide is under the control of a promoter that is active in eukaryotic cells.

Another embodiment encompasses a method of inhibiting a Bcl-2-associated disease comprising obtaining a polynucleotide that hybridizes to a Bcl-2-encoding polynucleotide, mixing the polynucleotide with a lipid to form a polynucleotide/lipid association, and administering said association to a cell. The cell may be a cancer cell, such as a follicular lymphoma cell. This method may employ a polynucleotide comprising an oligonucleotide having a length of between about 8 and about 50 bases. The lipid may form a comprising a liposome. If so, the liposome may further encapsulate the polynucleotide.

This embodiment also includes methods wherein the contacting takes place in an animal, such as a human. For example, the composition may be delivered to said human in a volume of 0.50-10.0 ml per dose or in an amount of from about 5 to about 30 mg polynucleotide per m². It may also be administered three times per week for eight weeks.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein:





FIG. 1: Growth inhibition of lymphoid cells by liposomal-bcl-2-antisense oligonucleotides ("L-bcl-2"). Final concentrations of 3 $\mu\text{mol/L}$ (), 4 $\mu\text{mol/L}$ (), 5 $\mu\text{mol/L}$ () and 6 $\mu\text{mol/L}$ () of L-bcl-2 were added to Johnson, Jurkat, Raji and Daudi cells. After 5 days, the viability of the tumoral cells was measured by alamarBlue dye. Viability was expressed as percent of untreated cells.




FIG. 2: Non-specific toxicity in lymphoid cells at 6 $\mu\text{mol/L}$ of liposomal oligonucleotides. Empty liposomes () and two different liposomal control oligonucleotides ("L-control oligos") ( , ) were added to Johnson, Jurkat, Raji and Daudi cells at 6 $\mu\text{mol/L}$ final concentration. After 5 days, the viability of the tumoral cells was measured by alamar blue dye. Viability was expressed as percent of untreated cells.

FIG. 3: Western blot analysis of Bcl-2 protein in the four cell lines. Johnson, Jurkat, Daudi and Raji cells were lysed in sample buffer and normalized for total protein content. Twenty-five grams of total protein was loaded in each lane. The membranes were incubated with hamster anti-human bcl-2 monoclonal antibody. In Johnson cells, a cell line bearing the t(14;18) translocation, overexpression of Bcl-2 protein is observed.

In Jurkat and Raji cells, which lack the t(14;18) translocation, expression of Bcl-2 is low. In Daudi cells, Bcl-2 expression is not observed.

FIG. 4A & FIG. 4B: Specific inhibition of Bcl-2 protein in Johnson and Jurkat cells by L-bcl-2



5 **FIG. 4A:** 1×10^5 Johnson cells/mL in 3 mL were treated with 3 and 4 $\mu\text{mol/L}$ of L-bcl-2 or L-control oligos. After 3 days of culture, protein-containing lysates were prepared and 5 μg of total protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were cut into sections and incubated with antibodies specific for either Bcl-2 or Actin (left). To estimate the inhibition of bcl-2 protein, data
10 were quantified by scanning densitometry and expressed as ratio of Bcl-2:Actin (right). L-bcl-2:(); L-control:().





FIG. 4B: 1×10^5 Jurkat cells/mL in 3 mL were treated with 3 and 4 $\mu\text{mol/L}$ of L-bcl-2 or L-control oligonucleotides. After 3 days of culture, protein-containing lysates were prepared and 20 μg of total protein were subjected to SDS-PAGE and transferred to
15 nitrocellulose membranes. Blots were cut into sections and incubated with antibodies specific for either Bcl-2 or Actin (left). To estimate the inhibition of bcl-2 protein, data were quantified by scanning densitometry and expressed as ratio of Bcl-2:Actin (right). L-bcl-2:(); L-control:().

FIG. 5: Western blot analysis of Bax protein in the four cell lines. Johnson,
20 Jurkat, Daudi and Raji cells were lysed in sample buffer and normalized for total protein content. Fifty μg of total protein was loaded in each lane. The membranes were incubated with rabbit anti-human Bax polyclonal antibody.

FIG. 6A & FIG. 6B: Bcl-2/Bax ratio decreases in Johnson cells by L-bcl-2.

FIG. 6A: 1×10^5 Johnson cells/mL in 3 mL were treated with 2, 3 and 4 $\mu\text{mol/L}$
25 of L-bcl-2 or L-control oligos. After 3 days of culture, protein-containing lysates were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Blots were cut into




sections, and incubated with antibodies specific for either Bax or Actin. This experiment was made using the same lysates obtained in the experiment shown in FIG. 4A and FIG. 4B.

5 **FIG. 6B:** Data were quantified by scanning densitometry and expressed as ratio of Bcl-2:Bax. L-bcl-2:(); L-control oligo:().

10 **FIG. 7:** DNA fragmentation in Johnson cells incubated with L-Bcl-2. Johnson cells were incubated with 4 $\mu\text{mol/L}$ of L-bcl-2 and two L-control oligos. After 3 days of incubation, DNA was extracted, electrophoresed through a 2% agarose gel and stained with ethidium bromide. Lanes 1, untreated cells; lane 2, cells treated with L-control (scrambled) oligo; lane 3, cells treated with L-control (random) oligo; lane 4, cells treated with L-bcl-2.

FIG. 8A & FIG. 8B: Apoptotic Johnson cells incubated with L-bcl-2.

15 **FIG. 8A:** Fluorescent photograph of Johnson cells dyed with the DNA-binding dye, acridine orange, after 3 days of incubation with 5 $\mu\text{mol/L}$ of L-bcl-2 (right) or without liposomal oligonucleotides ("L-OS") (left).

FIG. 8B: Apoptotic index of Johnson cells treated with 4 and 5 μM of L-bcl-2 () , L-control oligo () or empty liposomes (). Apoptotic index= (total no. of cells with apoptotic nuclei/total no. of cell counted) x 100%.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

20 *bcl-2* is an oncogene with tumorigenic potential due to its capacity to block programmed cell death. The present invention employs liposomal antisense oligodeoxynucleotides to inhibit the production of Bcl-2 so that tumor cells can regain the capacity to enter programmed cell death. The present invention may also be used to treat hematologic malignancies, both leukemias and lymphomas, including follicular and
25 nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias;

solid tumors like those associated with breast, prostate and colon cancer; and immune disorders, which are associated with Bcl-2 expression.

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The present invention relates to antisense oligonucleotides and polynucleotides directed to portions of the *bcl-2* gene and their use in the treatment of Bcl-2 related diseases. A specific type of cancer that may be treated by the methods of the presents of the present invention is FL. Over 90% of follicular lymphoma patients have a t(14;18) translocation which results in the translocation of the *bcl-2* gene from its normal location in chromosome 18 to the immunoglobulin heavy chain gene locus on chromosome 14. In consequence, the *bcl-2* gene is under the influence of the immunoglobulin heavy chain enhancer, and the Bcl-2 protein is overexpressed. Since *bcl-2* is an oncogene with tumorigenic potential due to its capacity to block programmed cell death, a potential therapy for these follicular lymphomas is to inhibit the production of the Bcl-2 protein. The present invention hopes to succeed where other approaches have failed by incorporating antisense oligonucleotides specific for the translation initiation site of the Bcl-2 mRNA into liposomes to inhibit the production of Bcl-2 protein.

In particular, it is contemplated that by using these antisense molecules, either alone or in conjunction with other antisense molecules, it is possible to effectively treat FL, and possibly other cancers. For example, this invention teaches that liposomal *bcl-2* antisense oligonucleotides (L-*bcl-2*) inhibit the growth of FL cells and in others cells which overexpress Bcl-2 protein. The oligo- or polynucleotides themselves, or expression vectors encoding therefor, may be employed. The preferred method of delivering these nucleic acids is via liposomes. The invention, in its various embodiments, is described in greater detail, below.

A. Polynucleotides and Oligonucleotides

The term "antisense" is intended to refer to polynucleotide molecules complementary to a portion of a Bcl-2 RNA, or the DNA's corresponding thereto. "Complementary" polynucleotides are those which are capable of base-pairing according

to the standard Watson-Crick complementarity rules. That is, the larger purines will base pair with the smaller pyrimidines to form combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T) in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA. Inclusion of less common bases such as inosine, 5-methylcytosine, 6-methyladenine, hypoxanthine and others in hybridizing sequences does not interfere with pairing.

Targeting double-stranded (ds) DNA with polynucleotides leads to triple-helix formation; targeting RNA will lead to double-helix formation. Antisense polynucleotides, when introduced into a target cell, specifically bind to their target polynucleotide and interfere with transcription, RNA processing, transport, translation and/or stability. Antisense RNA constructs, or DNA encoding such antisense RNA's, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject.

The intracellular concentration of monovalent cation is approximately 160 mM (10 mM Na⁺; 150 mM K⁺). The intracellular concentration of divalent cation is approximately 20 mM (18 mM Mg⁺; 2 mM Ca⁺⁺). The intracellular protein concentration, which would serve to decrease the volume of hybridization and, therefore, increase the effective concentration of nucleic acid species, is 150 mg/ml. Constructs can be tested *in vitro* under conditions that mimic these *in vivo* conditions.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. It is contemplated that the most effective antisense constructs for the present invention will include regions complementary to the mRNA start site. One can readily test such constructs simply by testing the constructs *in vitro* to determine whether levels of the target protein are affected. Similarly, detrimental non-specific inhibition of protein synthesis also can be measured by determining target cell viability *in vitro*.

As used herein, the terms "complementary" or "antisense" mean polynucleotides that are substantially complementary over their entire length and have very few base mismatches. For example, sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide for thirteen or fourteen positions out of fifteen. Naturally, sequences which are "completely complementary" will be sequences which are entirely complementary throughout their entire length and have no base mismatches.

Other sequences with lower degrees of homology also are contemplated. For example, an antisense construct which has limited regions of high homology, but also contains a non-homologous region (*e.g.*, a ribozyme) could be designed. These molecules, though having less than 50% homology, would bind to target sequences under appropriate conditions.

The polynucleotides according to the present invention may encode a *bcl-2* gene or a portion of that gene that is sufficient to effect antisense inhibition of protein expression. The polynucleotides may be derived from genomic DNA, *i.e.*, cloned directly from the genome of a particular organism. In other embodiments, however, the polynucleotides may be complementary DNA (cDNA). cDNA is DNA prepared using messenger RNA (mRNA) as template. Thus, a cDNA does not contain any interrupted coding sequences and usually contains almost exclusively the coding region(s) for the corresponding protein. In other embodiments, the antisense polynucleotide may be produced synthetically.

It may be advantageous to combine portions of the genomic DNA with cDNA or synthetic sequences to generate specific constructs. For example, where an intron is desired in the ultimate construct, a genomic clone will need to be used. The cDNA or a synthesized polynucleotide may provide more convenient restriction sites for the remaining portion of the construct and, therefore, would be used for the rest of the sequence.

5 The DNA and protein sequences for Bcl-2 are published in literature by Tsujimoto and Croce (1986) (SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6, & SEQ ID NO:7) which is incorporated herein by reference. It is contemplated that natural variants of Bcl-2 exist that have different sequences than those disclosed herein. Thus, the present invention is not limited to use of the provided polynucleotide sequence for Bcl-2 but, rather, includes use of any naturally-occurring variants. Depending on the particular sequence of such variants, they may provide additional advantages in terms of target selectivity, *i.e.*, avoid unwanted antisense inhibition of related transcripts. The present invention also encompasses chemically synthesized mutants of these sequences.

10 As stated above, although the antisense sequences may be full length genomic or cDNA copies, or large fragments thereof, they also may be shorter fragments, or "oligonucleotides," defined herein as polynucleotides of 50 or less bases. Although shorter oligomers (8-20) are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of base-pairing. For example, both binding affinity and sequence specificity of an oligonucleotide to its complementary target increase with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45 or 50 base pairs or larger may be used. While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence.

20 In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

25 As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in both DNA and RNA. Ribozymes can either be targeted

directly to cells, in the form of RNA oligonucleotides incorporating ribozyme sequences, or introduced into the cell as an expression vector encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense polynucleotide. Ribozyme sequences also may be modified in much the same way as described for antisense polynucleotide. For example, one could incorporate non-Watson-Crick bases, or make mixed RNA/DNA oligonucleotides, or modify the phosphodiester backbone.

Alternatively, the antisense oligo- and polynucleotides of the present invention may be provided as mRNA via transcription from expression constructs that carry nucleic acids encoding the oligo- or polynucleotides. Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid encoding an antisense product in which part or all of the nucleic acid sequence is capable of being transcribed. Typical expression vectors include bacterial plasmids or phage, such as any of the pUC or BluescriptTM plasmid series or, as discussed further below, viral vectors adapted for use in eukaryotic cells.

In preferred embodiments, the nucleic acid encodes an antisense oligo- or polynucleotide under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, or introduced synthetic machinery, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately

7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters
5 lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a
10 number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter,
15 it appears that individual elements can function either cooperatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding the inhibitory peptide is not believed to be important, so long as it is capable of expressing the peptide in the targeted cell. Thus, where a human cell is
20 targeted, it is preferable to position the nucleic acid coding the inhibitory peptide adjacent to and under the control of a promoter that is active in the human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat
25 can be used to obtain high-level expression of various proteins. The use of other viral or mammalian cellular or bacterial phage promoters which are well-known in the art to

achieve expression of peptides according to the present invention is contemplated as well, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of an antisense oligo- or polynucleotide can be optimized. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of an inhibitory protein. For example, a nucleic acid under control of the human PAI-1 promoter results in expression inducible by tumor necrosis factor. Tables 1 and 2 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of antisense constructs. This list is not intended to be exhaustive of all the possible elements involved in the promotion of expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding an NF-IL6 inhibitory peptide in an expression construct (Table 1 and Table 2). Additionally

- any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) also could be used to drive expression of a nucleic acid according to the present invention. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain
- 5 bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

TABLE 1

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β -Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DR α
β -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase <i>I</i>
Metallothionein

PROMOTER
Collagenase
Albumin Gene
α -Fetoprotein
τ -Globin
β -Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α 1-Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma

0972531.043197
26TET0T292490

PROMOTER
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

TABLE 2

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β -Interferon	poly(rI)X poly(rc)
Adenovirus 5 <u>E2</u>	Ela
c-jun	Phorbol Ester (TPA), H ₂ O ₂
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester (TPA)
Tumor Necrosis Factor	PHA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

5 In certain embodiments of this invention, the delivery of a nucleic acid in a cell may be identified *in vitro* or *in vivo* by including a marker in the expression construct. The marker would result in an identifiable change to the transfected cell permitting easy identification of expression. Enzymes such as herpes simplex virus thymidine kinase (*tk*) (eukaryotic) or chloramphenicol acetyltransferase (CAT) (prokaryotic) may be employed.

10 One also may include a polyadenylation signal to effect proper polyadenylation of the transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Examples include the SV40, globin or adenovirus polyadenylation signals. Also contemplated as an element of the expression cassette is a terminator. These elements can serve to enhance message levels and to minimize read through from the cassette into other sequences.

B. Lipid Formulations

15 In a preferred embodiment of the invention, the antisense oligo- or polynucleotides and expression vectors may be associated with a lipid. A polynucleotide associated with a lipid may be encapsulated in the aqueous interior of a liposome, interspersed within the lipid bilayer of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the polynucleotide, entrapped in a liposome, complexed with a liposome, dispersed in a solution containing a lipid, mixed with a lipid, combined with a lipid, contained as a suspension in a lipid, contained or 20 complexed with a micelle, or otherwise associated with a lipid. The lipid or lipid/oligonucleotide associated compositions of the present invention are not limited to any particular structure in solution. For example, they may be present in a bilayer structure, as micelles, or with a "collapsed" structure. They may also simply be interspersed in a solution, possibly forming aggregates which are not uniform in either 25 size or shape.

Lipids are fatty substances which may be naturally occurring or synthetic lipids. For example, lipids include the fatty droplets that naturally occur in the cytoplasm as well

as the class of compounds which are well known to those of skill in the art which contain long-chain aliphatic hydrocarbons and their derivatives, such as fatty acids, alcohols, amines, amino alcohols, and aldehydes. An example is the lipid dioleoylphosphatidylcholine.

5 "Liposome" is a generic term encompassing a variety of single and multilamellar lipid vehicles formed by the generation of enclosed lipid bilayers or aggregates. Liposomes may be characterized as having vesicular structures with a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when
10 phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991). However, the present invention also encompasses compositions that have different structures in solution than the normal vesicular structure. For example, the lipids may
15 assume a micellar structure or merely exist as nonuniform aggregates of lipid molecules. Also contemplated are lipofectamine-nucleic acid complexes.

Liposome-mediated polynucleotide delivery and expression of foreign DNA *in vitro* has been very successful. Wong *et al.* (1980) demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo,
20 HeLa and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection.

In certain embodiments of the invention, the lipid may be associated with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989).
25 In other embodiments, the lipid may be complexed or employed in conjunction with nuclear non-histone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the lipid may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression vectors have been successfully employed in transfer

and expression of a polynucleotide *in vitro* and *in vivo*, then they are applicable for the present invention. Where a bacterial promoter is employed in the DNA construct, it also will be desirable to include within the liposome an appropriate bacterial polymerase.

Phospholipids are used for preparing the liposomes according to the present invention and can carry a net positive charge, a net negative charge or are neutral. Diacetyl phosphate can be employed to confer a negative charge on the liposomes, and stearylamine can be used to confer a positive charge on the liposomes.

Lipids suitable for use according to the present invention can be obtained from commercial sources. For example, dimyristyl phosphatidylcholine ("DMPC") can be obtained from Sigma Chemical Co., dicetyl phosphate ("DCP") is obtained from K & K Laboratories (Plainview, NY); cholesterol ("Chol") is obtained from Calbiochem-Behring; dimyristyl phosphatidylglycerol ("DMPG") and other lipids may be obtained from Avanti Polar Lipids, Inc. (Birmingham, Ala.). Stock solutions of lipids in chloroform or chloroform/methanol can be stored at about -20°C. Preferably, chloroform is used as the only solvent since it is more readily evaporated than methanol.

Phospholipids from natural sources, such as egg or soybean phosphatidylcholine, brain phosphatidic acid, brain or plant phosphatidylinositol, heart cardiolipin and plant or bacterial phosphatidylethanolamine are preferably not used as the primary phosphatide, *i.e.*, constituting 50% or more of the total phosphatide composition, because of the instability and leakiness of the resulting liposomes.

Liposomes used according to the present invention can be made by different methods. The size of the liposomes varies depending on the method of synthesis. A liposome suspended in an aqueous solution is generally in the shape of a spherical vesicle, having one or more concentric layers of lipid bilayer molecules. Each layer consists of a parallel array of molecules represented by the formula XY, wherein X is a hydrophilic moiety and Y is a hydrophobic moiety. In aqueous suspension, the concentric layers are arranged such that the hydrophilic moieties tend to remain in contact

with an aqueous phase and the hydrophobic regions tend to self-associate. For example, when aqueous phases are present both within and without the liposome, the lipid molecules may form a bilayer, known as a lamella, of the arrangement XY-YX. Aggregates of lipids may form when the hydrophilic and hydrophobic parts of more than one lipid molecule become associated with each other. The size and shape of these aggregates will depend upon many different variables, such as the nature of the solvent and the presence of other compounds in the solution.

Liposomes within the scope of the present invention can be prepared in accordance with known laboratory techniques. In one preferred embodiment, liposomes are prepared by mixing liposomal lipids, in a solvent in a container, *e.g.*, a glass, pear-shaped flask. The container should have a volume ten-times greater than the volume of the expected suspension of liposomes. Using a rotary evaporator, the solvent is removed at approximately 40°C under negative pressure. The solvent normally is removed within about 5 min. to 2 hours, depending on the desired volume of the liposomes. The composition can be dried further in a desiccator under vacuum. The dried lipids generally are discarded after about 1 week because of a tendency to deteriorate with time.

Dried lipids can be hydrated at approximately 25-50 mM phospholipid in sterile, pyrogen-free water by shaking until all the lipid film is resuspended. The aqueous liposomes can be then separated into aliquots, each placed in a vial, lyophilized and sealed under vacuum.

In the alternative, liposomes can be prepared in accordance with other known laboratory procedures: the method of Bangham *et al.* (1965), the contents of which are incorporated herein by reference; the method of Gregoriadis, as described in *DRUG CARRIERS IN BIOLOGY AND MEDICINE*, G. Gregoriadis ed. (1979) pp. 287-341, the contents of which are incorporated herein by reference; the method of Deamer and Uster (1983), the contents of which are incorporated by reference; and the reverse-phase evaporation method as described by Szoka and Papahadjopoulos (1978). The

aforementioned methods differ in their respective abilities to entrap aqueous material and their respective aqueous space-to-lipid ratios.

P-ethoxy oligonucleotides, nucleases resistant analogues of phosphodiester, are preferred because they are stable in serum and effectively transported into the cellular cytoplasm. In a preferred embodiment, the lipid dioleoylphosphatidylcholine is employed. However other lipids such as other phosphatidylcholines, phosphatidylglycerols, and phosphatidylethanolamines may also be useful. Nuclease-resistant oligonucleotides were mixed with lipids in the presence of excess t-butanol. The mixture was vortexed before being frozen in an acetone/dry ice bath. The frozen mixture was lyophilized and hydrated with Hepes-buffered saline (1 mM Hepes, 10 mM NaCl, pH 7.5) overnight, and then the liposomes were sonicated in a bath type sonicator for 10 to 15 min. The size of the liposomal-oligonucleotides typically ranged between 200-300 nm in diameter as determined by the submicron particle sizer autodilute model 370 (Nicomp, Santa Barbara, CA).

25 C. Alternative Delivery Systems

by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - *gag*, *pol*, and *env* - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the *gag* gene, termed Ψ , functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a Bcl-2 antisense construct is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the *gag*, *pol* and *env* genes but without the LTR and Ψ components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing an inserted DNA, together with the retroviral LTR and Ψ sequences, is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

Adenoviruses: Human adenoviruses are double-stranded DNA tumor viruses with genome sizes of approximate 36 kB. As a model system for eukaryotic gene expression, adenoviruses have been widely studied and well characterized, which makes them an attractive system for development of adenovirus as a gene transfer system. This group of viruses is easy to grow and manipulate, and they exhibit a broad host range *in vitro* and *in*

vivo. In lytically infected cells, adenoviruses are capable of shutting off host protein synthesis, directing cellular machineries to synthesize large quantities of viral proteins, and producing copious amounts of virus.

The E1 region of the genome includes E1A and E1B which encode proteins responsible for transcription regulation of the viral genome, as well as a few cellular genes. E2 expression, including E2A and E2B, allows synthesis of viral replicative functions, *e.g.* DNA-binding protein, DNA polymerase, and a terminal protein that primes replication. E3 gene products prevent cytolysis by cytotoxic T cells and tumor necrosis factor and appear to be important for viral propagation. Functions associated with the E4 proteins include DNA replication, late gene expression, and host cell shutoff. The late gene products include most of the virion capsid proteins, and these are expressed only after most of the processing of a single primary transcript from the major late promoter has occurred. The major late promoter (MLP) exhibits high efficiency during the late phase of the infection (Stratford-Perricaudet and Perricaudet, 1991).

As only a small portion of the viral genome appears to be required *in cis* adenovirus-derived vectors offer excellent potential for the substitution of large DNA fragments when used in connection with cell lines such as 293 cells. Ad5-transformed human embryonic kidney cell lines (Graham, *et al.*, 1977) have been developed to provide the essential viral proteins *in trans*.

Particular advantages of an adenovirus system for delivering foreign proteins to a cell include (i) the ability to substitute relatively large pieces of viral DNA by foreign DNA; (ii) the structural stability of recombinant adenoviruses; (iii) the safety of adenoviral administration to humans; and (iv) lack of any known association of adenoviral infection with cancer or malignancies; (v) the ability to obtain high titers of the recombinant virus; and (vi) the high infectivity of adenovirus.

Further advantages of adenovirus vectors over retroviruses include the higher levels of gene expression. Additionally, adenovirus replication is independent of host

gene replication, unlike retroviral sequences. Because adenovirus transforming genes in the E1 region can be readily deleted and still provide efficient expression vectors, oncogenic risk from adenovirus vectors is thought to be negligible (Grunhaus & Horwitz, 1992).

5 In general, adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1, and yet still retains its competency for infection. Sequences encoding relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3
10 regions are capable of carrying up to 10 kB of foreign DNA and can be grown to high titers in 293 cells (Stratford-Perricaudet and Perricaudet, 1991). Surprisingly persistent expression of transgenes following adenoviral infection has also been reported.

Other Viral Vectors as Expression Constructs. Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses
15 such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpes viruses may be employed. They offer several attractive features for various mammalian cells (Friedman *et al.*, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988; Horwich *et al.*, 1990).

20 With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. *in vitro* studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich *et al.*, 1990). This suggested that large portions of the genome could be replaced with foreign
25 genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang *et al.* (1991) recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding sequences. It was

cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang *et al.*, 1991).

5 Non-viral Methods. Several non-viral methods for the transfer of expression vectors into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and van der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and
10 Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979) and lipofectamine-DNA complexes, cell sonication (Fecheimer *et al.*, 1987), gene bombardment using high velocity microprojectiles (Yang *et al.*, 1990), polycations and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for *in vivo* or *ex vivo* use.

15 In one embodiment of the invention, the expression construct may simply consist of naked recombinant vector. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. For example, Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice
20 demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding an Bcl-2 construct may also be transferred in a similar manner *in vivo*.

25 Another embodiment of the invention for transferring a naked DNA expression vector into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high

voltage discharge to generate an electrical current, which in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded *in vivo* (Yang *et al.*, 1990; Zelenin *et al.*, 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ. DNA encoding a Bcl-2 construct may be delivered via this method.

D. Pharmaceutical Compositions and Routes of Administration

Where clinical application of liposomes containing antisense oligo- or polynucleotides or expression vectors is undertaken, it will be necessary to prepare the liposome complex as a pharmaceutical composition appropriate for the intended application. Generally, this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate buffers to render the complex stable and allow for uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount of the antisense expression vector encapsulated in a liposome as discussed above, further dispersed in pharmaceutically acceptable carrier or aqueous medium. Such compositions also are referred to as inocula. The phrases "pharmaceutically" or "pharmacologically acceptable" refer to compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate.

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the

therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

5 Solutions of therapeutic compositions can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

10 The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain 10 mg, 25 mg, 50 mg or up to about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable
15 carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like.

Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous vehicles include fluid and nutrient
20 replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations are suitable for oral administration. Oral formulations
25 include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills,

capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

The therapeutic compositions of the present invention may include classic pharmaceutical preparations. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Topical administration would be particularly advantageous for the treatment of skin cancers, to prevent chemotherapy-induced alopecia or other dermal hyperproliferative disorder. Alternatively, administration may be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For treatment of conditions of the lungs, the preferred route is aerosol delivery to the lung. Volume of the aerosol is between about 0.01 ml and 0.5 ml. Similarly, a preferred method for treatment of colon-associated disease would be via enema. Volume of the enema is between about 1 ml and 100 ml.

An effective amount of the therapeutic composition is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the protection desired.

Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting the dose include the physical and clinical state of the patient, the route of administration, the intended goal of treatment (alleviation of symptoms *versus* cure) and the potency, stability and toxicity of the particular therapeutic substance. For the instant application, it is envisioned that the

amount of therapeutic peptide included in a unit dose will range from about 5-30 mg of polynucleotide.

E. Examples

EXAMPLE 1: Synthesis of Oligonucleotides.

5 Nuclease-resistant p-ethoxy oligonucleotides, non-ionic phosphodiester analogs, were purchased from Oligo Therapeutics (Willsonville, OR). An oligonucleotide specific for the translation initiation site of human Bcl-2 mRNA :
5'CAGCGTGCGCCATCCTTC^{3'} (SEQ ID NO:1) was used as antisense oligonucleotide. Two different control oligonucleotides were used: 5'ACGGTCCGCCACTCCTTCCC^{3'}
10 (SEQ ID NO:2) (scrambled version of Bcl-2 antisense oligonucleotide) and the random sequence 5'CTGAAGGGCTTCTTCC^{3'} (SEQ ID NO:3).

EXAMPLE 2: Preparation of liposomal oligonucleotides (L-OS)

P-ethoxy-oligonucleotides dissolved in distilled water were added to phospholipids (Avanti Polar Lipids, Alabaster, AL) in the presence of excess tert-butanol.
15 The mixture was frozen in a dry ice/acetone bath, lyophilized overnight and finally hydrated with HEPES buffered saline (1 mmol/L Hepes and 10 mmol/L NaCl) at a final oligonucleotide concentration of 0.1 mmol/L. Liposomal oligonucleotides (L-OS) were sonicated for 12 minutes in a bath-type sonicator. The average diameter of the particles was 100 nm ± 50 nm as determined in a NICOMP particle sizing system (Santa Barbara,
20 CA).

EXAMPLE 3: Oligonucleotide Inhibition of Protein Expression

Cell Lines

Johnson cells, a human transformed FL cell line bearing the t(14;18) translocation which overexpresses Bcl-2 protein, were used. Raji and Jurkat cells, a human Burkitt lymphoma cell line and a human acute T cell leukemia cell line, respectively, were also used. Both lines express the Bcl-2 protein but they lack the t(14;18) translocation. Daudi cells, a human Burkitt lymphoma cell line which does not express the Bcl-2 protein, was used as a negative control cell line. Johnson, Raji and Jurkat cells were grown in RPMI 1640 media (GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Daudi cells were grown in RPMI 1640 media supplemented with 20% heat-inactivated FBS.

Delivery of L-OS to cells

Ten thousand cells/well were seeded in a 96-well plate in 0.1 mL of the respective medium. Cells were incubated with L-OS at final concentration of 2 to 8 $\mu\text{mol/L}$ at 37° C in a 5% CO₂ incubator. Each experiment was done in triplicate and repeated at least 3 times.

Cell viability assay

The viability of the neoplastic cells was measured by the alamarBlue dye (Alamar, Sacramento, CA). After 5 days of incubation with L-OS, 40 μL of cells/well were aliquoted and added to 140 μL of fresh medium. Twenty μL of alamarBlue dye were added to each well. After incubation for 12 hours at 37°C, the plates were read directly on a microplate reader (Molecular Devices, CA) at 570 and 595 nm. The difference in absorbance between 570 and 595 nm was taken as the overall absorbance value of the cells. All experiments were analyzed by t-test in which the viabilities of the cells treated with the L-OS were compared with those of the untreated controls.

Western Blots for Bcl-2 and Bax protein

One hundred thousand cells/well were seeded in a 6-well plate in 3 mL of the respective medium, treated with 2, 3 and 4 $\mu\text{mol/L}$ of L-OS and incubated at 37°C. Untreated cells were also maintained in culture. Samples were removed on day 3 after the addition of the L-OS and lysed in 100 μL of lysis buffer (1% Triton, 150 mmol/L NaCl and 25 mmol/L Tris pH 7.4) at 0°C for 30 minutes. After centrifugation at 12,000 x g for 10 minutes, the supernatants were recovered and normalized for total protein content (5 $\mu\text{g/lane}$ of Johnson cells lysate and 20 $\mu\text{g/lane}$ of Jurkat cells lysate for Bcl-2 analysis, and 25 $\mu\text{g/lane}$ of Johnson cells for Bax analysis). The lysates were mixed with sample buffer containing 1% of sodium dodecyl sulfate (SDS) and 1% 2- β -mercaptoethanol and boiled for 5 minutes. SDS-PAGE was run on 10% polyacrylamide gels, electrophoretically transferred to nitrocellulose membranes and blocked in 10% non-fat dry milk. Filters were cut in 2 portions: the bottom section was incubated with the 6C8 hamster anti-human-Bcl-2 monoclonal antibody or rabbit anti-human-Bax polyclonal antibody (Hockenbery et al), and the top section was incubated with mouse anti-actin monoclonal antibody (Amersham) at room temperature for 2 hours. After washing and incubation with a peroxidase-labeled antihamster (Kirkegaard & Perry laboratories), antirabbit (Santa Cruz) or antimouse (Amersham) secondary antibody, blots were developed by enhanced chemiluminescence system (ECL, Amersham). To estimate the inhibition of Bcl-2 protein and the ratio of Bcl-2/Bax proteins, densitometric scans were performed on western blots on a Gilford Response Gel Scanner (CIBA Corning, Medfield, MA). Area integration of absorbance peaks at 500 nm was used to determine the ratio of Bcl-2:Actin and Bcl-2:Bax proteins.

Analysis of apoptosis

To qualitatively determine the internucleosomal DNA cleavage associated with apoptosis, DNA fragmentation analysis by agarose gel electrophoresis was performed (Duke *et. al*, 1991). In brief, 1×10^6 Johnson cells were cultured in 10 mL of medium in a 75 sq. cm tissue culture flask, treated with 4 $\mu\text{mol/L}$ of L-OS, and incubated at 37°C.

Untreated cells were also maintained in culture. Samples were removed on day 3 after treatment, washed in PBS and pellet by centrifuging 10 minutes at 200 x g. The pellets were lysed in 0.5 mL of lysis buffer (10 mmol/L Tris pH 7.4, 1 mmol/L EDTA pH 8.0 and 0.2% Triton X-100) and fragmented DNA were separated from intact chromatin by microcentrifuging for 10 minutes at 13,000 x g. DNA of the supernatants was precipitated in 0.7 mL ice-cold isopropanol overnight at -20°C, resuspended in 30 µL of TE buffer (10 mmol/L Tris pH 7.4, 1 mmol/L EDTA pH 8.0) and incubated in 10 µL of RNase (10 µg/mL solution) at 60°C for 1 hour. Twenty µL of sample per well were electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining.

To quantitatively determine the extent of apoptosis, we used the fluorescent DNA-binding dye acridine orange (Duke *et. al*, 1991). Briefly, 5 µmol/L final concentration of L-OS were added to 1×10^5 cells/well plated in a 24-well plate in 1 mL of medium. After 3 days of incubation at 37°C, the cells were washed with PBS and resuspended at 1×10^6 cells/mL. Twenty-five µL of cell suspension were mixed with 1 µL of acridine orange dye (100 µg/mL, Sigma Chemicals, St. Louis, MO) and observed in a fluorescent microscope. The percentage of apoptotic cells (apoptotic index) was obtained using a hemocytometer. Apoptotic index= (total no. of cells with apoptotic nuclei/total no. of cells counted) x 100%.

Effect of L-bcl-2-antisense oligonucleotides ("L-bcl-2") on lymphoma cell growth

Five days after the addition of L-bcl-2 to the cells, the viability of tumoral cells was assessed. Cell growth was inhibited in a concentration-dependent manner in Johnson cells, which bear the t(14;18) translocation and express very high levels of Bcl-2. A concentration of 6 µmol/L L-bcl-2 resulted in complete loss of viability of Johnson cells within 5 days (FIG. 1). Similar dose-dependent decrease in cell viabilities could be seen in three separate experiments. In contrast, after treatment with 6 µmol/L()L-bcl-2, the viabilities of Jurkat, Raji and Daudi cells decreased by only 23%, 0% and 35%, respectively (FIG. 1).

Effect of liposomal control oligonucleotides (L-control oligos) on lymphoma cell growth

Two control oligonucleotides were used to determine the specificity of the inhibition observed. When L-control oligos or empty liposomes were added to Johnson cells, cell growth inhibition was not observed. Jurkat, Raji and Daudi cells were also treated with L-control oligos and empty liposomes. Non-specific toxicity could be observed when greater than 6 $\mu\text{mol/L}$ of L-OS were used, but not with empty liposomes (FIG. 2).

Selective inhibition of Bcl-2 protein by L-bcl-2-antisense oligonucleotides

In order to determine whether the cytotoxic effect of L-bcl-2 in Johnson cells was caused by a decrease in Bcl-2 protein, the Bcl-2 protein expression in these cells after treatment with L-bcl-2 as well as the effects of L-bcl-2 in the other cell lines which overexpress Bcl-2 protein (FIG. 3). was also determined.

When Johnson cells were treated with 2, 3 and 4 $\mu\text{mol/L}$ of L-bcl-2, the ratios of Bcl-2/Actin protein were inhibited by 28, 57 and 64%, respectively. Bcl-2 protein expression was not inhibited in cells treated with the same doses of L-control oligos.

When Jurkat cells were treated with 3 and 4 $\mu\text{mol/L}$ of L-bcl-2, the ratios of Bcl-2/Actin protein were inhibited by 44% and 50%, respectively. Bcl-2 protein was not significantly inhibited when the same doses of L-control oligos were used (FIG. 4).

The ratios of Bcl-2/Bax protein in Johnson and Jurkat cells before and after treatment with L-bcl-2 and L-control oligos were also determined. Johnson, Raji and Daudi cells expressed Bax protein but Jurkat cells did not express (FIG. 5). When Johnson cells were treated with 2, 3 and 4 $\mu\text{mol/L}$ of L-bcl-2, the ratio of Bax/Actin was not modified, but the ratio of Bcl-2/Bax decreased by 10%, 40% and 50%, respectively. These protein ratios were unmodified after treatment with the same doses of L-control oligos (FIG. 6).

Inhibition of Bcl-2 protein leads to apoptosis in the FL cells

Whether the growth inhibitory effects seen in Johnson cells may be related to induction of apoptosis was also studied. After 3 days of incubation with L-bcl-2, the typical internucleosomal DNA degradation pattern was observed, whereas cells incubated with L-control oligos did not show the internucleosomal DNA pattern (FIG. 7). Subsequently, the quantity of apoptosis by acridine orange was assessed. After 3 days of exposure to 4 and 5 $\mu\text{mol/L}$ of L-bcl-2, apoptotic cells were evident. The apoptotic index of untreated Johnson cells was 3% while that of Johnson cells treated with 4 and 5 $\mu\text{mol/L}$ of L-bcl-2 were 43% and 61%, respectively. Significant increase in apoptotic index was not seen in cells treated with liposomal control oligonucleotides or empty liposomes (FIG. 8).

L-bcl-2 selectively downregulates the expression of Bcl-2 protein and cell growth in a dose-dependent manner

The inhibition in cell growth was seen only in the FL cell line which bears the t(14;18) translocation, while cell growth inhibition was not seen in cell lines that lack the Bcl-2 expression (Daudi cells) or the t(14;18) translocation (Raji and Jurkat cells). There was no non-specific toxicity in Johnson cells exposed to two different control oligonucleotides. The growth inhibitory effects could be observed starting at a concentration of 3 $\mu\text{mol/L}$ of L-bcl-2, and the inhibitory effects were maximal at 6 $\mu\text{mol/L}$ concentration. One of the mechanisms by which L-bcl-2 exerts growth inhibition in Johnson cells might be through induction of apoptosis, since treated cells showed a typical DNA internucleosomal degradation pattern, and an increased apoptotic index as measured by acridine orange. By day 3, 61% of Johnson cells treated with 5 $\mu\text{mol/L}$ of L-bcl-2 were in apoptosis as compared with 15% of cells treated with L-control oligos. Apoptosis was not observed in the other cell types.

Thus, the inhibition of Bcl-2 protein leads to cell growth inhibition in cells that are dependent on the presence of Bcl-2 protein for maintaining viability. Gene transfer

experiments have provided evidence that Bcl-2 plays an important role in maintaining lymphoid cell survival in vitro, although other autocrine growth factors may also be involved (Vaux *et. al*, 1988; Reed *et. al*, 1990b; Blagosklonny and Neckers, 1995). Using phosphorothiorate antisense oligonucleotides, Cotter and co-workers observed growth inhibition in DoHH2 cell line which has the t(14;18) translocation and overexpress Bcl-2 protein, but not in FC11 cell line which overexpresses Bcl-2 protein without the t(14;18) translocation (Cotter *et. al*, 1994). Cells that overexpress Bcl-2 and lack the t(14;18) translocation may need an apoptotic stimulus, like growth factor deprivation or treatment with chemotherapeutic drugs, to be driven into apoptosis and growth arrest (Reed, 1995). Antisense oligonucleotides may be used to reverse the chemotherapeutic resistance of those cells that also overexpress high levels of Bcl-2 without the t(14;18) translocation (Kitada *et. al*, 1994).

Bax, a promoter of apoptotic cell death, may be a common partner involved in heterodimerization and regulation of other Bcl-2 family members function (Sedlak *et. al*, 1995). It has been suggested that, the equilibrium in the formation of Bcl-2:Bax heterodimers and Bax:Bax homodimers appears to be central in the molecular regulation of apoptosis (Yin *et. al*, 1994). Moreover, in a recent study, the ratio of Bcl-2:Bax correlated with cell death in IL-3-dependent FL5.12 cells. When approximately half or more of Bax was heterodimerized with Bcl-2, apoptosis was inhibited (Yang *et. al*, 1995). The apoptotic death observed in Johnson cells, after incubation with L-bcl-2, could be due to decrease in Bcl-2:Bax ratio and formation of more Bax:Bax homodimers.

Another explanation is that other oncogenes and tumor suppressor genes such as C-MYC and p53 may be involved in the survival of the other cell lines. C-MYC, for example, is typically expressed in Burkitt lymphomas and in some transformed FL (McDonnell and Korsmeyer, 1991). Mutations of p53 gene, a suppressor gene involved in numerous human tumors, may also be involved in these cell lines; p53 gene encodes a DNA-binding protein that functions at least in part as a transcription factor to induce cell cycle arrest and apoptosis by upregulation of Bax (Vogelstein and Kinzler, 1992;

Miyashita *et. al*, 1994; Miyashita and Reed, 1995). Probably, inhibition of Bcl-2 expression alone is not enough to induce apoptosis and growth inhibition in cells other than Johnson cells.

5 A decrease in the ratio of Bcl-2/Bax by L-bcl-2 forms the basis for a molecular approach to follicular lymphoma therapy.

EXAMPLE 4: *In vivo* Testing

10 In an initial round of *in vivo* trials, inventors can use a mice model of human cancer with the histologic features and metastatic potential resembling tumors seen in humans and treat these animals with lipid-associated oligo- or polynucleotide compositions to examine the suppression of tumor development.

15 These studies are based on the discovery that *bcl-2* antisense oligonucleotides associated with lipids inhibit the production of the Bcl-2 protein and the growth of t(14;18) translocation bearing cells (Examples 1-3). The Examples above further show that these lipid formulations inhibit the growth of *bcl-2*-related cancer cells. The current example uses lipid-associated poly- and oligonucleotide formulations, either alone or in combination with chemotherapeutic drugs, to provide a useful preventive and therapeutic regimen for patients with *bcl-2*-overexpressing cancers.

20 Mice of a suitable cancer model (*see, e.g.*, McDonnell, 1993) will be treated with doses of lipid-associated oligo- or polynucleotide compositions starting at 8-10 weeks of age or approximately 25 g in weight. The mice used may be transgenic mice bearing the t(14;18) translocation, or they may be nude or SCID mice that were implanted intraperitoneally with human FL cell lines. Several combinations and concentrations of these formulations will be tested. Three groups of mice will be used: untreated mice (or mice injected with buffer only), mice injected with liposomal antisense oligos, and mice
25 injected with liposomal control oligos. The animals will be injected intravenously with

liposomal oligos twice a week. The doses will range between 0-15 mg of liposomal oligos per kg of mouse in weight. The treatments will be from 6 to 8 weeks.

5 The effect of the lipid-associated oligonucleotide compositions on the development of FL tumors will be compared with the control group by measuring tumor size, mouse survival, B cell hyperplasia, and Bcl-2 expression. It is predicted that, unlike the control groups of mice that will develop tumors, the testing group of mice will have decreased Bcl-2 expression, B cell hyperplasia, and tumor size, as well as prolonged survival. The group treated with liposomal control oligos should have no such effects.

EXAMPLE 5: Clinical Trials

10 This example is concerned with the development of human treatment protocols using the lipid-associated oligo- and polynucleotide compositions. These lipid formulations will be of use in the clinical treatment of various *bcl-2*-overexpressing cancers and diseases in which transformed or cancerous cells play a role. Such treatment will be particularly useful tools in anti-tumor therapy, for example, in treating patients
15 with FL. This treatment will also be useful in treating other conditions that are mediated by *bcl-2* over-expression and resistant to conventional regimens and treatments such as hematologic malignancies, both leukemias and lymphomas, including follicular and nonfollicular lymphomas, chronic lymphocytic leukemia, and plasma cell dyscrasias; solid tumors like those associated with breast, prostate and colon cancer; and immune
20 disorders.

The various elements of conducting a clinical trial, including patient treatment and monitoring, will be known to those of skill in the art in light of the present disclosure. The following information is being presented as a general guideline for use in
25 establishing lipid-associated oligo-and polynucleotide compositions alone or in combinations with anti-cancer drugs in clinical trials.

Candidates for the phase 1 clinical trial will be patients on which all conventional therapies have failed. Liposomal Bcl-2 antisense oligos will be administered to them

intravenously on a tentative weekly basis. To monitor disease course and evaluate the anti-tumor responses, it is contemplated that the patients should be examined for appropriate tumor markers every month. To assess the effectiveness of the drug, the following parameters will be monitored: tumor size and bone marrow infiltration of the cancer cells. Tests that will be used to monitor the progress of the patients and the effectiveness of the treatments include: physical exam, X-ray, blood work and other clinical laboratory methodologies. In addition, peripheral blood and bone marrow samples will be drawn to assess the modification of the target protein expression. The doses given in the phase 1 study will be escalated as is done in standard phase 1 clinical phase trials, i.e. doses will be escalated until maximal tolerable ranges are reached.

Clinical responses may be defined by acceptable measure. For example, a complete response may be defined by complete disappearance of evidence of cancer cells for at least 2 months. Whereas a partial response may be defined by a 50% reduction of cancer cells for at least 2 months.

15 EXAMPLE 6: Human Treatment and Clinical Protocols

This example describes a protocol to facilitate the treatment of *bcl-2*-mediated diseases using lipid-associated oligo- or polynucleotide compositions alone or in combination with anti-cancer drugs.

Typically, patients that are candidates for treatment are those with FL although patients with hematologic malignancies, both leukemias and lymphomas; solid tumors like those associated with breast, prostate and colon cancer; and immune disorders may also be treated with the methods of this invention. The typical course of treatment will vary depending upon the individual patient and disease being treated in ways known to those of skill in the art. For example, a patient with FL might be treated in eight week cycles, although longer duration may be used if no adverse effects are observed with the patient, and shorter terms of treatment may result if the patient does not tolerate the treatment as

hoped. Each cycle will consist of between 20 and 35 individual doses spaced equally, although this too may be varied depending on the clinical situation.

A patient presenting a *bcl-2*-mediated condition, like FL, may be treated using the following protocol. Patients may, but need not, have received previous chemo-, radio- or gene therapeutic treatments. Optimally the patient will exhibit adequate bone marrow function (defined as peripheral absolute granulocyte count of $> 2,000/\text{mm}^3$ and platelet count of $100,000/\text{mm}^3$, adequate liver function (bilirubin 1.5mg/dl) and adequate renal function (creatinine 1.5mg/dl).

The over-expression of *bcl-2* is typically monitored before, during, and after the therapy. A composition of the present invention is typically administered orally or parenterally in dosage unit formulations containing standard, well known non-toxic physiologically acceptable carriers, adjuvants, and vehicles as desired. The term parenteral as used herein includes subcutaneous injections, intravenous, intramuscular, intra-arterial injection, or infusion techniques. The lipid-associated oligo- or polynucleotide compositions may be delivered to the patient before, after or concurrently with the other anti-cancer agents.

A typical treatment course may comprise about six doses delivered over a 7 to 21 day period. Upon election by the clinician the regimen may be continued with six doses every three weeks or on a less frequent (monthly, bimonthly, quarterly etc.) basis. Of course, these are only exemplary times for treatment, and the skilled practitioner will readily recognize that many other time-courses are possible.

To kill *bcl-2*-overexpressing cancer cells using the methods and compositions described in the present invention one will generally contact a target cell with the lipid-associated formulations described previously. These compositions will be provided in an amount effective to kill or inhibit the proliferation of the cell.

Regional delivery of the lipid-associated formulations will be an efficient method for delivering a therapeutically effective dose to counteract the clinical disease.

Alternatively systemic delivery may be appropriate. The therapeutic composition of the present invention may be administered to the patient directly at the site of the tumor. This is in essence a topical treatment of the surface of the cancer. The volume of the composition should usually be sufficient to ensure that the entire surface of the tumor is
5 contacted by the lipid-associated oligo- or poly-nucleotide composition.

In one embodiment, administration simply entails injection of the therapeutic composition into the tumor. In another embodiment, a catheter is inserted into the site of the tumor and the cavity may be continuously perfused for a desired period of time.

Clinical responses may be defined by acceptable measure. For example, a
10 complete response may be defined by complete disappearance of evidence of cancer cells for at least 2 months. Whereas a partial response may be defined by a 50% reduction of cancer cells for at least 2 months.

Of course, the above-described treatment regimes may be altered in accordance with the knowledge gained from clinical trials such as those described in Example 5.
15 Those of skill in the art will be able to take the information disclosed in this specification and optimize treatment regimes based on the clinical trials described in the specification.

H. References

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